Appendices XVII & XVIII
FDC Jan 2003

CHAPTER I.2.

GENERAL INFORMATION

1. PRELIMINARY REMARK

World mollusc production is adversely affected by several diseases and, given their severe impact on economic and socio-economic development in many countries, some diseases have become a primary constraint to the growth and sustainability of this sector. Diseases are also a major threat to aquaculture natural resources. Pathogen transfer via transfers of live molluscs has been a major cause of disease outbreaks and epizootics. Eleven significant diseases of molluscs are currently listed in the *International Aquatic Animal Health Code*. Information relevant to these diseases is provided in the following chapters of this *Diagnostic Manual for Aquatic Animal Diseases*. Recent scientific information available in taxonomic affiliation of *Mikrocytos roughleyi* and its relationship with *Bonamia* species has resulted in the re-definition of bonamiosis. Table 1. below shows how the chapters on mollusc diseases in this edition of the *Manual* correspond with those in the *Code*.

Table 1. *How the chapters on mollusc diseases in this edition of the* Diagnostic Manual for Aquatic Animal Diseases *correspond with those in the* International Aquatic Animal Health Code

Manual:	***Code:***
Bonamiosis:	infection with *Bonamia ostreae*
	infection with *Bonamia exitiosus*
	infection with *Mikrocytos roughleyi*
MSX disease:	infection with *Haplosporidium nelsoni*
Marteiliosis:	infection with *Marteilia refringens*
	infection with *Marteilia sydneyi*
Mikrocytosis:	infection with *Mikrocytos mackini*
Perkinsosis:	infection with *Perkinsus marinus*
	infection with *Perkinsus olseni/atlanticus*
SSO disease:	infection with *Haplosporidium costale*
Withering syndrome of abalone:	infection with *Candidatus* Xenohaliotis californiensis

2. SAMPLING

2.1. Sampling

A general approach to surveillance and sampling is given in the introductory chapter of this *Manual* entitled Requirements for surveillance for international recognition of freedom from infection. The sampling should be designed in order to enable detection, at a 95% confidence level, of pathogen carriers. The following section gives information relevant to sampling molluscs.

The individual disease chapters provide more detailed information and disease-specific data required for the design of surveillance and health certification schemes.

2.2. Specific recommendations for sampling molluscs

The timing and frequency of sampling should be determined by the cycle of infection by the pathogen and the prepatent period. An adequate time period should be allocated when sampling for seasonal diseases, for example infection with *Marteilia refringens* or *Haplosporidium nelsoni*, to ensure optimal detection. As pathogens may increase in intensity of infection associated with loss of host condition following spawning, post-spawning sampling is also recommended. Sampling periods must also take account of the transfer of juveniles and spats into outgrowing areas, and the transfer of adults for further fattening or relaying.

Samples should also cover a range of size groups, or target the most susceptible age group when this is known. During sampling, any molluscs showing abnormalities (abnormal growth, gaping valves, elevated or high mortality rates) should be selected. Diseases have subclinical stages of development that can escape detection using routine screening methodology. However, the probability of detection of infection may be increased by holding the bivalves in quarantine for a long period and subjecting them to stress (crowding, handling, temperature and salinity changes, etc.). To detect infection, species of molluscs that are more susceptible to infection should be examined histologically. For example, members of the Arcidae (*Arca, Barbatia*), Malleidae (*Malleus*), Isognomonidae (*Isognomon*), Chamidae (*Chama*) and Tridacnidae (*Tridacna*) tolerate high prevalence of *Perkinsus* infection and are good indicators of the presence of *Perkinsus*.

Prevalence of infection is an important factor affecting the chance of detection. When molluscs are to be moved from natural beds into a farm site or between natural beds in different zones, large numbers of bivalves may be sampled taking into account low prevalence of infection. For example, in Western Australia, *Marteilia sydneyi* and *Perkinsus* sp. occur in isolated beds untouched by humans at 0.1% prevalence.

For each zone, a number of sampling sites must be selected in the most practicable way so as to maximise the chances of detecting pathogens. The number of sites must be increased for large zones that contain several discrete areas of cultivation of the susceptible species. Account must be taken of parameters that have an effect on the development of the pathogenic agents, such as stocking density, water flow, and the developmental cycle of the molluscs.

It is an important requirement that the screening techniques used be the optimum methods available for detection of the disease agent in question, and that when the infection is present, it can be detected. For screening methods, sensitivity and specificity should have been assessed. This information has a strong influence on sample size.

For pathogen-specific details see the individual disease chapters of the *Manual*.

3. SHIPMENT OF SAMPLES

All sampled molluscs must be delivered to the approved diagnostic laboratory within 24 hours of sampling. The laboratory should be informed of the estimated time of arrival of the sample so the required materials to process the molluscs can be prepared before reception of samples.

Mollusc samples must be packed in accordance with current standards in order to keep them alive. If the sampling site is a long distance from the laboratory, moribund animals or those with foul-smelling tissues may be of little use for subsequent examination. Required samples should be shipped as soon as possible after collection from the water, in order to reduce air storage and possible mortality during transportation, especially for moribund diseased molluscs.

For samples that cannot be delivered live to the diagnostic laboratory, due to advanced stages of disease, long distance or slow transportation connections, etc., specimens should be fixed on site as recommended in the following sections of this chapter or individual disease chapters of this *Manual*. While this is suitable for, for example, subsequent histology or transmission electron microscopy examination, other techniques, such as fresh smears, tissue imprints, routine bacteriology, mycology or Ray's fluid thioglycollate culture of *Perkinsus* spp., cannot be performed. Diagnostic needs and sample requirements should be discussed with the diagnostic laboratory prior to collection of the sample.

Samples should be accompanied with background information, including the reason for submitting the sample (surveillance, abnormal mortality, abnormal growth, etc.), gross observations and associated environmental parameters, approximate prevalence and patterns of mortality, origin and nature of the molluscs (species, age, whether or not the samples are from local mollusc populations or stocks transferred from another site, date of transfer and source location, etc.). This information should identify possible changes in handling or environmental conditions that could be a factor in mortality in association, or not, with the presence of infectious agents.

4. MACROSCOPIC EXAMINATION

The gross observation of molluscs should target, as far as possible, animal behaviour, shell surface, inner shell and soft tissues.

It is often difficult to observe the behaviour of molluscs in open waters and often the shell no longer exists. However, close attention can be made of behaviour of molluscs in certain rearing facilities such as brood-stock in tanks and larvae in hatcheries. If signs are noted (e.g. pre-settlement of larvae on the bottom, food accumulation in tanks, signs of weakening, etc.), samples may be examined for gross signs, including observation under a dissecting microscope for abnormalities and deformities, fouling organisms, and fixed for further processing as recommended below. For adults and juveniles, signs of weakening may include gaping, accumulation of sand, mud and debris in the mantle and on the gills, mantle retraction away from the edge of the shell, decreased activity (scallop' swimming, clam' burrowing, abalone' grazing), etc. Open-water mortality should be monitored for patterns of losses and samples collected for further analysis. Environmental factors pre- and post-mortality should be recorded.

Even under culture conditions, shell of molluscs may not be clean and fouling organisms are normal colonists of mollusc shell surfaces. Organisms such as barnacles, limpets, sponges, polychaete worms, bivalve larvae, tunicates, bryozoans, etc., do not normally threaten health of molluscs. Culture systems, such as suspension and shallow water culture, can even increase exposure to fouling organisms and shells may become covered by other animals and plants. This can affect the health directly by impeding shell opening and closing or indirectly through competition for food resources. Signs of weakening associated with heavy fouling should be a cause for concern rather than fouling itself. Shell damages by boring organisms such as sponges and polychaete worms are usually benign, but under certain conditions may reach proportions that make the shell brittle or pierce through to the soft-tissues. This degree of shell damage can weaken the mollusc and render it susceptible to inter-current pathogen infections. Shell deformities (shape, holes in the surface), fragility, breakage or repair should be noted, but are not usually indicative of a disease concern. Abnormal coloration and smell, however, may indicate a possible soft-tissue infection that may need to be examined at a laboratory.

The molluscs must be opened carefully so as not to damage the soft tissues, in particular the mantle, gills, heart and digestive gland. The presence of fouling organisms on the inner shell surface is a clear indication of weakness. The inner surface of the shell is usually smooth and clean due to mantle and gill action. Perforation of the inner surface may occur, but can be sealed off by the deposition of additional conchiolin and nacre. This may result in formation of mud- or water-filled blisters. Blisters may also form over superficial irritants such as foreign bodies. The degree of shell perforation can be determined by holding the shell up to a strong light. Where abnormalities occurring within the matrix of the shell warrant further investigation, freshly collected specimens can be brought intact to the laboratory or fixed for subsequent decalcification, as required. The appearance of the soft-tissues is

frequently indicative of the physiological condition of the animal. Soft tissues should be examined for the presence of abscess lesions, pustules, tissue discoloration, pearls, oedema, overall transparency or wateriness, gill deformities, etc., and, when found in association with weak or dying animals, these abnormalities should be a cause for concern.

Abnormalities and lesions of the tissues should be noted and recorded, as well as any shell deformities, shell-boring organisms and conspicuous mantle inhabitants. Levels of tissue damage should be recorded and samples of affected and unaffected animals collected for laboratory examination as soon as possible.

5. EXAMINATION OF STOCKS WHERE ABNORMAL MORTALITY OCCURS

Abnormal mortality of molluscs is usually recognised as a sudden sizeable mortality that occurs in a short time between two observations or inspections of the stocks (for example, about 15 days in the case of facilities located in inter-tidal zone). In a hatchery, abnormal mortality is the failure of successive productions of larvae coming from different brood-stock. Given the broad spectrum of species, environments and culture conditions these definitions should be adapted when and where necessary.

Whenever abnormal mortality occurs in stocks of molluscs, an urgent investigation must be carried out to determine the aetiology.

The samples taken must be consistent with the requirements for surveillance provided in the *Manual*. The samples should also be preserved or fixed and stored in accordance with the procedures defined for histological and other appropriate presumptive and confirmatory methods.

Where and when available, unaffected or control molluscs should also be fixed for histological comparison with abnormal tissues. Whatever the fixative, it is essential that the shell be removed to allow easy ingress of the fixative. Bivalves and operculated gastropods can keep the shell shut against fixative until autolysis begins.

6. DIAGNOSTIC METHODS

Techniques applicable to molluscan pathogens are limited, and most of the investigations are based on histological and ultrastructural examinations. Classic serological methods cannot be used for diagnostic purposes because molluscs do not produce antibodies. Immunoassays using monoclonal antibodies or nucleic acid probes can be used for direct detection of certain pathogenic agents. A number of research teams and diagnostic laboratories have been engaged in developing DNA-based diagnostic techniques for mollusc pathogens. Given the development and potential for widespread application of these diagnostic techniques and the inherent problems currently associated with their use, the issue of validation is of the utmost importance.

Three levels of examination procedures are proposed in the following sections. Screening (surveillance) is routinely performed by histology. Histology is recommended as a standard screening method because it provides a large amount of information. It is particularly important because macroscopic examination usually gives no pathognomonic signs or solid indicative information. Also, mortality may be due to several pathogens or physiological problems, such as loss of condition following spawning, and this can only be determined using histology.

When abnormal mortality outbreaks occur, various presumptive diagnostic methods can be used in addition to histology, among which, tissue imprints, Ray's fluid thioglycollate medium (RFTM) culture or polymerase chain reaction (PCR) are likely to be used as recommended in the in the individual disease chapters. Such methods may provide advantages of quick and/or cheap procedures as an answer to suspicion of infection with a given pathogen.

When a pathogen is encountered during screening or mortality outbreaks, electron microscopy and/or molecular probes should be used for specific identification, if available. Some of the OIE notifiable

diseases for molluscs cover pathogenic agents belonging to one or more species of the same genus. Specific reagents designed to detect certain listed agents are recommended in the following chapters, to be used to confirm histological examination results and/or to give a species-specific diagnosis where available.

6.1. Histological techniques

Because of the generic use of histology in diagnostic procedures for diseases of molluscs, a detailed technical guideline is provided in this chapter.

Histology is a technique that is used to study the structure of cells and tissues under light microscopy. Tissue preparation involves different steps, including tissue fixation, dehydration, impregnation and embedding of samples, preparation of sections, staining and mounting of slides.

Live moribund animals or freshly dead (within minutes) animals provide the optimum conditions under which to collect tissues. A standard section should be taken through the digestive gland, to include the gills, mantle and palps, where possible. Alternatively for large specimens, several sections should be taken to include all the important tissues.

• 6.1.1. Tissue fixation

The role of the fixative is to maintain the morphology of the tissues as close to *in-vivo* morphology as possible and to prevent post-sampling necrosis. Recommended fixatives used for the study of marine molluscs are Davidson's solution and Carson's solution for large specimens. For smaller specimens, GHF or other glutaraldehyde fixatives may be used and are compatible for electron microscopy use. The ratio of fixative to tissue volume should be at least 10:1 to ensure good fixation.

Davidson's solution:

Sea water	1200 ml
95% Alcohol	1200 ml
38% Formaldehyde	300 ml
Glycerol	400 ml
Glacial acetic acid	10% (add extemporaneously)

Carson's solution:

$NaH_2PO_4 2H_2O$	23.8 g
NaOH	5.2 g
Distilled water	900 ml
40% Formaldehyde	100 ml

Adjust the pH to 7.2–7.4

There is no universal fixative and choice should be made taking into account later use of fixed material as well as practical aspects of fixative use (price, component availability, etc.). Davidson's solution is an excellent choice for preserving the structure of the tissues. In addition, tissue sections fixed in Davidson's solution can be stained later by different

histochemical methods, as well as *in-situ* hybridisation with DNA probes. For this purpose, over-fixation (over 24–48 hours) should be avoided. Carson's solution may not be as good as Davidson's solution for histological analysis. Nevertheless it does allow good preservation of the ultrastructure and may be used to preserve samples for later study by electron microscopy. Because electron microscopy may be a valuable adjunct in diagnosing or confirming infections in molluscs, fixation of some samples (especially smaller samples) using glutaraldehyde, as described in Section 6.2 of this chapter, may be considered. Otherwise, material fixed in Carson's solution, and shown to contain adequate levels of targeted pathogens or abnormalities, can be refixed in glutaraldehyde. It is recommended that part of the mollusc be fixed in Davidson's solution while the other part be fixed in Carson's solution for further investigation. This should be done in order to ensure fixation of all tissues/organs in the two fixatives. If neither are available, 10% buffered formalin made up with filtered seawater is adequate. Within each country, the molluscan aquaculture industry must agree on the most effective way of ensuring adequate fixation.

- **6.1.2. Dehydration, impregnation and embedding of the samples**

The embedding of the samples in paraffin requires several steps during which the water contained in the tissues is progressively replaced, first by alcohol, then by xylene or equivalent less toxic clearing solution, and lastly by paraffin.

After having fixed the samples in Carson's or Davidson's solution, they are transferred through graded alcohols (70–95 [v/v]) before final dehydration in absolute ethanol. The alcohol contained in the tissues is next eliminated by immersing them in xylene. The tissues are then impregnated with paraffin, which is soluble in xylene, at 60°C. These steps may be all carried out automatically using a machine.

Blocks are produced by letting the tissues cool in moulds filled with paraffin on a cooling table; cooling and moisturising are essential to section cutting.

- **6.1.3. Preparation of the sections**

After the blocks have been cooled on a cold plate, which allows the paraffin to solidify, histological sections of about 2–3 µm are cut using a microtome. The sections are recovered on histological slides, drained and dried overnight at 60°C. Drying the samples at this temperature allows the excess moisture to be eliminated and thus the sections adhere to the slides.

- **6.1.4. Staining and mounting the slides**

Before staining, the paraffin is removed from the sections by immersing them in xylene or equivalent less toxic clearing solution for 10–20 minutes. This is repeated once and then the solvent is eliminated by immersion in two successive absolute ethanol baths for 10-minute periods each and rehydrated by immersion in a bath of tap water for 10 minutes. Different topographical or histochemical staining techniques can then be performed.

When topographical staining with haematoxylin–eosin (H&E) is used, (haematoxylin or equivalent) nuclear and basophilic structures stain a blue to dark purple colour, the endoplasmic reticulum stains blue, while the cytoplasm takes on a grey colour. The acid dye eosin stains the other structures pink. This staining technique is simple and reproducible and, although it only allows a limited differentiation of cell structures, it is possible to detect any abnormalities in tissue and cellular structure. Other techniques may be applied to demonstrate particular structures or features as required (e.g. trichrome for connective tissue and cytoplasmic granules).

6.2. Transmission electron microscopy methods

Because of the very frequent use of transmission electron microscopy in confirmatory identification of pathogens in diagnostic procedures for diseases of molluscs, detailed technical guidelines are provided in this chapter for indication.

Fixation for electron microscopy should be done immediately before fixation for histology. Only samples taken rapidly from live animals will be of any use. The preparation of samples for electron microscopy involves the following steps: tissue fixation, decalcification of the samples (when necessary), dehydration, impregnation and embedding of the samples, preparation and counterstaining of the sections.

- **6.2.1. Tissue fixation**

  For tissues that are to be examined by electron microscopy, it is important that the fixation be performed correctly in order to cause as little damage as possible to the ultrastructure. The specimens are cut such that their width does not exceed 3–4 mm. This small size allows the various solutions to penetrate rapidly into the sample.

  Fixation of the samples is carried out directly in 3% glutaraldehyde for 1 hour. Fixation for longer periods leads to membranous artefacts. The samples are washed in buffer three times, then fixed in 1% osmic acid and washed twice again in buffer. Various formulations of glutaraldehyde fixative and buffers work equally well.

  In order to cause as little damage as possible to the ultrastructure, the samples are treated with solutions that have an osmolarity close to that of the tissues. Thus, mollusc tissues are treated with solutions with an osmolarity of around 1000 mOsm. The osmolarity of the solutions is adjusted with NaCl. As mollusc tissues are nearly iso-osmotic with seawater, it is possible to make the glutaraldehyde up with 0.22 µm filtered seawater, and use the filtered seawater for subsequent washes.

  If the samples have been previously fixed and stored in Carson's solution, they must be washed several times in a bath of buffer before fixation with 3% glutaraldehyde.

- **6.2.2. Dehydration, impregnation and embedding of the samples**

  The samples are dehydrated in successive baths of ethanol: 70% ethanol once, 95% ethanol twice, absolute ethanol three times. The dehydration is completed by two baths of propylene oxide, which allows the subsequent impregnation with Epon or other resin.

  The samples are impregnated progressively. After a first bath in a mixture of polypropylene oxide–Epon (50/50), the samples are placed in a bath of Epon. The longer the incubation, the better the impregnation of the tissues.

  Embedding is carried out by placing the samples in moulds filled with Epon resin. A label identifying the sample is included in each block and the blocks are then placed at 60°C (the temperature at which Epon resin polymerises) for 48 hours.

- **6.2.3. Preparation of the sections and the counterstaining**

  The blocks are cut to appropriate sizes with a razor blade and the sections are then cut using an ultramicrotome. Semi-thin sections (0.5–1 µm) are cut and placed on glass slides. These will be used to control the quality of the samples by light microscopy and to find the areas of interest on the section.

  The semi-thin sections are stained at 90–100°C with 1% toluidine blue solution. After drying, the slides are mounted under cover-slips with a drop of synthetic resin and observed under the light microscope.

Ultrathin sections 80–100 nm thick are placed on mesh copper grids for electron microscopy analysis. Uranyl acetate and lead citrate are used to counterstain the ultrathin sections.

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Appendix XVIII

CHAPTER I.3.

GENERAL INFORMATION

SUMMARY

1. NOTIFIABLE AND OTHER SIGNIFICANT DISEASES OF CRUSTACEANS

Crustaceans are adversely affected by a number of diseases. This is especially evident in penaeid shrimp from aquaculture. All of the crustacean diseases that have significant social or economic notoriety are infectious diseases. The crustacean diseases and their aetiological agents that are included in the *International Aquatic Animal Health Code* (the *Code*) have a restricted geographical range, have no therapeutic remedies or treatments, are potentially excludable, and are of significant social and economic importance. The list of crustacean diseases considered for notification and certification currently consists of three *Diseases Notifiable to the OIE* and five *Other Significant Diseases.* Seven of these eight crustacean diseases are listed by the OIE because of the size and importance of the penaeid shrimp aquaculture industry. Therefore, the principles and methods discussed in this chapter will, of necessity, emphasise the penaeid shrimp.

The OIE listed crustacean diseases, the nature of their respective aetiological agents, and their principal hosts are:

- **Notifiable Diseases of Crustaceans:**

  Taura syndrome (viral/penaeid shrimp)
  White spot disease (viral/penaeid shrimp and other decapod crustaceans)
  Yellowhead disease (viral/penaeid shrimp)

- **Other Significant Diseases of Crustaceans:**

  Tetrahedral baculovirosis (*Baculovirus penaei*) (viral/penaeid shrimp)
  Spherical baculovirosis (*Penaeus monodon*-type baculovirus) (viral/penaeid shrimp)
  Infectious hypodermal and haematopoietic necrosis (viral/penaeid shrimp)
  Crayfish plague *(Aphanomyces astaci)* (fungal/freshwater crayfish)
  Spawner-isolated mortality virus disease (viral/penaeid shrimp)

2. DIAGNOSTIC METHODS

The methods available for diagnosis of the above-listed diseases include the traditional methods of morphological pathology (direct light microscopy, histopathology, and electron microscopy), bioassay methods with susceptible indicator hosts, and molecular methods (gene probes and polymerase chain reaction [PCR]). While tissue culture is considered to be a standard tool in medical, veterinary, and fish diagnostic laboratories, it has yet to be developed as a usable, routine diagnostic tool for crustacean pathogens. Clinical chemistry has not become a routinely used diagnostic tool by crustacean pathologists.

2.1. Diagnostic methods for diseases of crustaceans

As of the date of the drafting of this section of the *Manual*, the available diagnostic methods that may be selected for diagnosis of the OIE listed crustacean diseases or detection of their aetiological agents are based on:

- **Gross and clinical signs**
- **Direct bright-field, phase-contrast or dark-field microscopy with whole stained or unstained tissue wet-mounts, tissue squashes, and impression smears; and wet-mounts of faecal strands**
- **Histology of fixed specimens**
- **Bioassays of suspect or asymptomatic carriers using a highly susceptible host (life stage or species) as the indicator for the presence of the pathogen**
- **Transmission or scanning electron microscopy**
- **Antibody-based tests for pathogen detection using immune sera (polyclonal antibodies) or monoclonal antibodies (MAbs)**
- **Molecular methods**

  DNA probes in dot-blot hybridisation assays directly with fresh tissue samples or with extracted DNA

  DNA probes or RNA probes for *in situ* hybridisation assays with histological sections of fixed tissues

  PCR and reverse-transcription (RT)-PCR for direct assay with fresh tissue samples or with extracted DNA or RNA.

The detailed procedures for each of the available methods (screening, presumptive, and confirmatory) for diagnosis of each of the OIE listed crustacean diseases are outlined in the respective sections of this *Manual.*

There is a paucity of antibody-based diagnostic tests available for the pathogens that cause crustacean diseases. As crustaceans do not produce antibodies, antibody-based diagnostic tests are limited in their application to pathogen detection. While a number of antibody-based diagnostic methods have been developed and are described in the literature, these were developed with mouse or rabbit antibodies generated to viruses purified from infected hosts. Because crustacean viruses cannot be routinely produced in tissue culture, purified virus from infected hosts must be used to produce antibody. This has severely limited the development and availability of this diagnostic tool. The recent application of MAb technologies to this problem has begun to provide a few antibody-based tests. MAbs are available for three of the OIE listed crustacean diseases (for Taura syndrome virus [TSV], infectious hypodermal and haematopoietic necrosis virus [IHHNV], and white spot syndrome virus [WSSV]). Antibody based diagnostic kits/reagents for TSV and WSSV infections are currently available from a commercial source.

Molecular methods have been developed and some methods are in widespread use for the detection of many of the viral, bacterial, and protozoan pathogens of the penaeid shrimp. DNA-based detection methods are readily available from the literature and some are available in kit form from commercial sources for the OIE notifiable pathogens TSV, WSSV, and yellowhead disease virus (YHV/GAV), and for IHHNV, *Penaeus monodon*-type

baculovirus (MBV), *Baculovirus penaei* (BP), and spawner-isolated mortality virus (SMV). PCR or RT-PCR methods are available for several of these viruses and some are in routine use by certain sectors of the crustacean aquaculture industry. For all the OIE listed viral pathogens, specific DNA probes tagged with nonradioactive labels are either reported in the literature or available commercially for application in dot-blot formats with haemolymph or tissue extracts, or for use with routine histological sections using *in situ* hybridisation.

Despite the growing dependence of the shrimp aquaculture industry on DNA-based diagnostic methods, none of the tests that are available from commercial sources or reported in the literature has been validated using controlled field trials. Likewise, there are few formal accreditation or certification programmes yet in place to assure that test results from technicians and laboratories are indeed accurate and the tests properly controlled. There is a growing need to standardise and validate the DNA-based diagnostic methods and the laboratories that use them. Standardisation of DNA-based diagnostic methods is almost inherent in the nature of the tests – that is, a specific DNA probe or a specific set of primers that is used to demonstrate the presence or absence of a unique DNA or RNA sequence does not vary from batch to batch. Hence, with proper controls, these DNA-based methods are readily standardised. The implementation of a formal programme by appropriate international agencies or professional societies is needed to validate new diagnostic methods and to periodically review the accreditation and certification of diagnosticians and diagnostic laboratories. The establishment of regional reference laboratories for DNA-based diagnostic methods of penaeid shrimp/prawn pathogens would fit well into such a programme with the goal of making these methods uniform, reliable, and readily applicable to disease control and management strategies for viral diseases of cultured penaeids.

3. SAMPLING

There are at least three purposes for which crustacean stocks may be sampled with regard to the OIE listed crustacean pathogens. These are: 1) surveillance; 2) stock or facility 'certification'; and 3) disease diagnosis. The number and type of samples to be taken for analysis varies greatly according to which of these purposes applies.

3.1. Diagnosis in disease situations

In clinical disease episodes, carefully selected quality specimens with representative lesions should be obtained from live or moribund crustaceans. Every effort should be made to sample those specimens for diagnosis that are representative of the disease(s) that is (are) affecting the crustacean stock of interest, and that are moribund or clinically diseased. Collection of dead specimens should be avoided. When cultured or wild crustacean stocks are presenting clinical signs of an active disease that are consistent with, or suggestive of, any one of the OIE listed crustacean diseases, care should be taken to ensure that the samples collected are preserved appropriately for the anticipated diagnostic tests (see sample preservation section for recommended methods).

The recommended minimum numbers of specimens to collect for diagnostic testing are 100 for the larval stages of most crustaceans; 50 for the postlarval stages; and 10 for juveniles and adults. Sample numbers may be greater if clinically diseased specimens are readily apparent and collected. Nonetheless, these recommended 'minimum' sample numbers are provided as guidelines, and it must be emphasised that carefully selected, quality specimens are far more valuable (and cost-effective) diagnostic specimens than dozens or hundreds of specimens taken at random to 'fill out' the sample.

3.2. Diagnosis in asymptomatic crustaceans

When samples are to be taken for surveillance, for testing of asymptomatic carriers of previous disease epizootics, for 'certification' of specific pathogen free (SPF) status, or for freedom from a particular disease within a country, zone, or facility the sample size to be taken should be determined using methods based on the provisions in the chapter on Requirements for Surveillance for International Recognition of Freedom from Infection. For surveillance and certification purposes for OIE listed diseases, the samples taken for diagnostic tests at any given aquaculture site or from wild stocks should include the appropriate number of specimens from each lot to be tested according on the provisions in the chapter on Requirements for Surveillance. For the OIE listed diseases it is highly recommended that the scheduling of sampling be planned (i.e. by farm schedule, season, etc.) so that the particular life-stage(s) are sampled at a time when the pathogen of concern is most likely to be detected. This is especially important when the available diagnostic methods are dependent on simple microscopy or histological methods and do not include molecular methods. For the baculoviruses, BP and MBV larval and early postlarval stages are the most appropriate samples; for TSV, IHHNV, WSSV and YHV/GAV, juveniles and subadults provide the best samples; and for crayfish plague, juveniles and adults are suitable samples.

4. SAMPLE TYPE AND PRESERVATION

4.1. Samples for direct microscopy

Samples for direct microscopic examination should be examined as soon as possible after collection. Use live specimens whenever possible, or use fresh, chilled, or 10% buffered formalin-fixed specimens when live specimens are not practical. If an adequate field laboratory is available, it should be used to process and examine samples near the site of collection.

4.2. Samples for histology

Collect shrimp by whatever means are available with a minimum of handling stress. Transport the shrimp to the laboratory via a well oxygenated water-filled utensil. Supply adequate aeration to the container if the shrimp are to be left for a short period of time before actual fixation. For the study of presumably diseased shrimp, select those shrimp that are moribund, discoloured, displaying abnormal behaviour, or otherwise abnormal, except in the case of intentional random sampling for estimation of disease prevalence.

i) Have ready an adequate supply of fixative. A general rule is that a minimum of ten volumes of fixative should be used for one volume of tissue sample (i.e. a 10 g sample of shrimp would require 100 ml of fixative).

ii) Davidson's AFA (alcohol, formalin, acetic acid) fixative

Davidson's AFA fixative is recommended for most histological applications. The fixative is rapid, reduces autolytic changes in tropical crustaceans (i.e. the penaeid shrimp), and its acidic content decalcifies the cuticle. The formulation for Davidson's AFA is (for 1 litre):

330 ml 95% ethyl alcohol
220 ml 100% formalin* (a saturated 37–39% aqueous solution of formaldehyde gas)
115 ml glacial acetic acid**
335 ml tap water (for marine crustaceans, sea water may be substituted)

Store the fixative in glass or plastic bottles with secure caps at room temperature.

* Do not use previously made 10% formalin to prepare Davidson's AFA because the formalin content of the Davidson's AFA will be inadequate to provide satisfactory fixation.

** Do not substitute other acids, such as HCl, for acetic acid. Histological sections prepared from HCl-Davidson's solution are not suitable for routine haematoxylin and eosin histological staining.

iii) Nonacidic R-F ('RNA-friendly') fixative

For some applications where *in situ* assays are planned with MAbs or with cDNA probes for RNA viruses (TSV or YHV), Davidson's fixative may be too acidic or too harsh. For such specimens an alternative fixative, which has advantages over buffered formalin, has been developed. R-F fixative is not recommended for routine use in crustacean histology. This is because it does not penetrate or fix tissues rapidly, and neither does it decalcify the cuticle. Hence, its use may result in poorly fixed tissues that are difficult to section and stain.

The formulation of R-F fixative is (for 1 litre):

407 ml 95% ethyl alcohol
349 ml 100% formalin
222 ml tap water (for marine crustaceans, sea water may be substituted)
22 ml ammonium hydroxide (28–30% as NH_3)
pH ~6.0–7.0
Store the fixative in glass or plastic bottles with secure caps at room temperature.

iv) Fixation procedures with Davidson's AFA or R-F fixative

- *For larvae and postlarvae that are too small to be easily injected with fixative using a tuberculin syringe:* Using a fine mesh screen or a Pasteur pipette, select and collect specimens. Immerse shrimp selected for sampling directly in the fixative. Fix for 12–24 hours in fixative, then transfer to 50–70% ethyl alcohol for storage.

- *For larger postlarvae and very small juveniles that are too small to be injected:* Select and collect specimens as described in Section 3. Use a needle or fine-pointed forceps to incise the cuticle. Immerse shrimp selected for sampling directly in the fixative. Fix for 12–24 hours in fixative, then transfer to 50–70% ethyl alcohol for storage.

- *For larger postlarvae, juveniles, and adults:* Inject fixative (use 5–10% volume: weight) via needle and syringe (needle gauge dependent on shrimp size, i.e. 27 gauge needle for postlarvae and small juveniles) into the living shrimp.

  The hepatopancreas (HP) should be injected first and at two or more sites, with a volume sufficient to change the HP to a white to orange colour; then inject fixative into adjacent regions of the cephalothorax, into the anterior abdominal region, and into the posterior abdominal region.

  The fixative should be divided between the different regions, with the cephalothorasic region, specifically the HP, receiving a larger share than the abdominal region.

A good guide to insure adequate fixation is to inject an equivalent of 5–10% of the shrimp's (or other crustacean's) body weight; all signs of life should rapidly cease, and visible colour change should occur in the injected areas.

Immediately following injection, slit the cuticle, with dissecting scissors, from the sixth abdominal segment to the base of the rostrum, being particularly careful not to cut deeply into the underlying tissue. The incision in the cephalothorasic region should be just lateral to the dorsal midline, while that in the abdominal region should be approximately mid-lateral.

- *For shrimp (and most other crustaceans) larger than ~12 g:* After injection of fixative, the body should then be transversely bisected, at least once, just posterior to the abdomen/cephalothorax junction, and (optional) again mid-abdominally.
- *For very large crustaceans and crabs:* The organs of interest may be excised after injection of fixative. Completion of fixation of these tissue samples is then handled as outlined previously.

Following injection, incisions and bisection/trisection, or excision of key organs, immerse the specimen in the fixative (use 10:1 fixative:tissue ratio).

Allow fixation to proceed at room temperature for 24–72 hours depending on the size of shrimp (or crustacean) being preserved. Longer fixation times in Davidson's AFA may be used to thoroughly decalcify the shell of crabs, lobsters, crayfish, etc.

Following fixation, the specimens should be transferred to 50–70% ethyl alcohol, where they can be stored for an indefinite period.

Record a complete history of the specimens at the time of collection: gross observations on the condition of the shrimp (or other crustacean), species, age, weight, source (wild, or if culture pond or tank number, stock number, etc.), and any other pertinent information that may be needed at a later time.

The label should stay with the specimens in the same container during fixation, storage and transport to the laboratory. Always use No. 2 soft-lead pencil on water-resistant paper (plastic paper is recommended; never use ink or marking pens).

v) Transport and shipment of preserved samples

Because large volumes of alcohol should not be posted or shipped, the following methods are recommended: Remove the specimens from the 50% or 70% ethyl alcohol. For larvae, postlarvae, or small juveniles, use leak-proof, screw-cap plastic vials if available; if glass vials must be used, pack to prevent breakage. For larger specimens, wrap samples with white paper towels to completely cover (do not use raw cotton). Place towel-wrapped specimens in a sealable plastic bag and saturate with 70% ethyl alcohol. Insert the label and seal the bag. Place the bag within a second sealable bag. Multiple small sealable bags can again be placed within a sturdy, crush-proof appropriately labelled container for shipment (see Section 1.5 of the *Code* for details).

4.3. Preservation of samples for antibody, DNA probe dot-blot tests, or polymerase chain reaction

For routine diagnostic testing by PCR, RT-PCR or for dot-blot tests with DNA probes, samples must be prepared to preserve the pathogen's nucleic acid. Likewise, samples intended for testing with antibody-based methods must be preserved to retain reactive antigenic sites for the antibodies used.

4.3.1. Sample types

Samples selected for DNA-based or antibody-based diagnostic tests should be handled and packaged (in new plastic sample bags or bottles) with great care to minimise the

potential for cross contamination among the sample set taken from different (wild or farmed) stocks, from tanks, ponds, farms, etc. New plastic sample bags or bottles must be used. A water-resistant label, with the appropriate data filled out in No. 2 pencil, should be placed within each package or container for each sample set.

Some suitable methods for preservation and transport of samples taken for molecular or antibody-based tests are:

- *Live specimens:* These may be processed in the field or shipped to the diagnostic laboratory for testing.

- *Haemolymph:* This tissue is the preferred sample for certain molecular and antibody-based diagnostic tests. Samples may be collected by needle and syringe by cardiac puncture, from the hemocoel (i.e. the ventral sinus in penaeids), or from a severed appendage.

- *Iced or chilled specimens:* This is for specimens that can be transported to the laboratory for testing within 24 hours. Pack samples in sample bags surrounded by an adequate quantity of wet ice around the bagged samples in a Styrofoam™-insulated box and ship to the laboratory.

- *Frozen whole specimens:* Select live specimens according to the criteria listed in Section 3, quick freeze in the field using crushed dry-ice, or freeze in the field laboratories using a mechanical freezer at –20°C or lower temperature. Prepare and insert the label into the container with the samples, pack samples with an adequate quantity of dry-ice in a Styrofoam™-insulated box, and ship to the laboratory.

- *Alcohol-preserved samples:* In regions where the storage and shipment of frozen samples is problematic, 90–95% ethanol may be used to preserve, store, and transport certain types of samples. Whole crustaceans (any life stage provided the specimen is no larger than 2–3 g), excised tissues (i.e. pleopods) from large crustaceans, or haemolymph may be preserved in 90–95% ethanol, and then packed for shipment according to the methods described in Section 4.2.v.

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